

METHODS OF INHIBITING MUSCLE ATROPHY

10/018761

sub 2.1 This application claims priority of U.S. Provisional Application No.
60/142,857, filed July 7, 1999, the entirety of which is incorporated
5 by reference herein.

BACKGROUND OF THE INVENTION

A decrease in muscle mass, or atrophy, is associated with various
10 physiological and pathological states. For example, muscle atrophy can
result from denervation due to nerve trauma; degenerative, metabolic
or inflammatory neuropathy, e.g. Guillian-Barré syndrome; peripheral
neuropathy; or nerve damage caused by environmental toxins or drugs.
Muscle atrophy may also result from denervation due to a motor
15 neuropathy including, for example, adult motor neuron disease, such as
Amyotrophic Lateral Sclerosis (ALS or Lou Gehrig's disease); infantile
and juvenile spinal muscular atrophies; and autoimmune motor
neuropathy with multifocal conductor block. Muscle atrophy may also
result from chronic disease resulting from, for example, paralysis due
20 to stroke or spinal cord injury; skeletal immobilization due to trauma,
such as, for example, fracture, ligament or tendon injury, sprain or
dislocation; or prolonged bed rest. Metabolic stress or nutritional
insufficiency, which may also result in muscle atrophy, include *inter*
alia the cachexia of cancer and other chronic illnesses including AIDS,
25 fasting or rhabdomyolysis, and endocrine disorders such as disorders of
the thyroid gland and diabetes. Muscle atrophy may also be due to a
muscular dystrophy syndrome such as Duchenne, Becker, myotonic,
fascioscapulohumeral, Emery-Dreifuss, oculopharyngeal,
scapulohumeral, limb girdle, and congenital types, as well as the

dystrophy known as Hereditary Distal Myopathy. Muscle atrophy may also be due to a congenital myopathy, such as benign congenital hypotonia, central core disease, nemaline myopathy, and myotubular (centronuclear) myopathy. Muscle atrophy also occurs during the aging process.

Muscle atrophy in various pathological states is associated with enhanced proteolysis and decreased production of muscle proteins. Muscle cells contain lysosomal proteases and cytosolic proteases. The cytosolic proteases include Ca^{2+} -activated neutral proteases (calpains) and an ATP-dependent ubiquitin-proteasome proteolytic system. The lysosomal and cytosolic systems are capable of degrading muscle proteins *in vitro*, but less is known about their roles in proteolysis of muscle proteins *in vivo*. Some studies have reported that proteasome inhibitors reduce proteolysis in atrophying rat skeletal muscle (e.g. Tawa et al. (1997) *J. Clin. Invest.* 100:197), leading to suggestions that the ubiquitin-proteasome pathway has a role in the enhanced proteolysis. However, the precise mechanisms of proteolysis in atrophying muscle remain poorly characterized.

Ras is a signaling molecule, found in every cell in the body. It is activated by a variety of growth factors, including growth factors which signal tyrosine kinase receptors. Ras activation results in activation of several downstream signaling pathways, including the Raf/Mek/Erk pathway. Other potential downstream substrates of Ras include the following signaling molecules: PKC, Ral-GDS, KSR, and Rin-1 (Trends Genet 1999 Apr;15(4):145-9). Ras, Raf, Mek and Erk genes are well-conserved through evolution, and homologs can be found in eukaryotes from yeast to humans (Ras: Taparowsky E, et al. (1982)

Nature 300:762-5; Raf: Heidecker, et al. (1990) Mol. Cell. Biol. 10:2503; Samuels, et al. (1993) Mol. Cell. Biol. 13:6241; Morrison, et al. (1997) Curr. Opin. Cell Biol. 9:174; Mek: Crews CM, et al. (1992) Science 258:478-80; Erks: Boulton TG, et al. (1991) Cell 65:663-75).

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When a constitutively active mutant of Ras was expressed in cardiac muscle in transgenic mice, the animals were born with hypertrophic heart tissue (J Biol. Chem. 1995 Sep 29;270(39):23173-8). This suggested the possibility that Ras activation might lead to hypertrophy in skeletal muscle as well, and indicated that Ras activation might be an efficacious method to reverse skeletal muscle atrophy.

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However, it has been discovered in accordance with the present invention that proteolysis or decreased synthesis in muscle cells is actually increased by activation of cell signaling molecules in the Ras and downstream Raf/Mek/Erk/ pathways.

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SUMMARY OF THE INVENTION

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The present invention provides a method of inhibiting skeletal muscle atrophy, by inhibiting signaling pathways that lead to skeletal muscle atrophy. Agents that inhibit Ras activation, or which inhibit signaling molecules downstream of Ras, such as Raf/Mek/Erk, in the present method are useful for preventing or reducing atrophy and/or causing hypertrophy in skeletal muscle cells.

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the transgene, injection of the transfected ES cells into host blastocysts into pseudopregnant foster mother hosts, followed by embryonic development and birth of the transgenic mice. The constitutively active mutant form of Ras, Raf, Mek, or Erk nucleic acid is expressed in muscle cells of the transgenic mice to provide the constitutively active mutant form of the Ras, Raf, Mek or Erk protein in the muscle cells.

In another preferred embodiment, the transgenic mouse is made by injection of a vector comprising the transgene directly into the pronucleus of a fertilized egg, as described in Hogan et al., supra. The injected egg is inserted into pseudopregnant foster mother hosts, followed by embryonic development and birth of the transgenic mouse.

The presence of constitutively active mutant forms of Ras, Raf, Mek or Erk protein can also be assessed using antibodies specific for these proteins, or by antibodies specific for an epitope-tag which is genetically inserted into the vectors encoding these proteins.

In methods for the identification of an agent that inhibits muscle cell atrophy, the agent may be contacted with the muscle cell expressing constitutively active mutant forms of Ras, Raf, Mek or Erk by methods known in the art. For cells in culture or cells obtained from transgenic organisms, the cell may be contacted with the agent by, for example, direct application. The agent may be modified or contained in a delivery vehicle to facilitate entry into the cell. The agent may be isolated and purified, or it may present in a sample or composition to be subjected to further purification subsequent to a positive result in the present method. For example, the agent may be contained in a cell

It has been discovered in accordance with the present invention that when skeletal muscle cells are treated with a chemical which blocks signaling of the Mek kinase, which is downstream of Ras and Raf, the resultant cells appear hypertrophic in comparison to control cells.

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Accordingly, Applicants have discovered that inhibitors of Ras or the downstream signaling molecules Raf/Mek/Erk are useful for reducing or preventing atrophy in skeletal muscle cells. Further, such inhibitors may be used to decrease and/or prevent atrophy in mammals having a condition, such as those described herein, in which skeletal muscle atrophy is occurring. In a preferred embodiment, such inhibitors are those already proven to be pharmacological antagonists for Ras [Cancer Chemother Pharmacol. (1999) 43(1):50-8], or Raf/Mek/Erk, such as PD98059 [Proc. Natl. Acad. Sci. (1995) 92:7686] or farnesyl transferase (Chem. Biol. (1995) Dec 2(12):787-91). According to this embodiment, atrophying skeletal muscle cells, or vertebrate animals having a condition as described above in which muscle cells are atrophying, are treated with an inhibitor so as to prevent or decrease muscle cell atrophy. Such treatment may be utilized prophylactically prior to the onset of muscle atrophy or after such condition has manifested itself. Vertebrate animals include any species containing skeletal muscle and a backbone, and includes chickens, rodents, rabbits, dogs, cats, cows, horses, pigs, sheep, primates and humans, preferably humans.

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In another embodiment, inhibitors of Ras or the downstream signaling molecules Raf/Mek/Erk may be used to cause hypertrophy in skeletal muscle cells. Further, such inhibitors may be used to cause muscle hypertrophy in vertebrate animals having conditions, such as those described herein, in which skeletal muscle atrophy is anticipated. In

some settings, such as in animals farmed for meat production, such agents might be used to increase meat production. In a preferred embodiment, such inhibitors are those already proven to be pharmacological antagonists for Ras [Cancer Chemother Pharmacol. (1999) 43(1):50-8], or Raf/Mek/Erk, such as PD98059 [Proc.Natl. Acad. Sci. (1995) 92:7686] or farnesyl transferase transferase (Chem. Biol. (1995) Dec 2(12):787-91). According to this embodiment, atrophying skeletal muscle cells, or vertebrate animals having a condition as described above in which muscle cells are atrophying, are treated with an inhibitor so as to prevent or decrease muscle cell atrophy. Such treatment may be utilized prophylactically prior to the onset of muscle atrophy or after such condition has manifested itself. Alternatively, such treatment may be used to induce skeletal muscle hypertrophy.

The invention further provides for therapeutic compositions comprising a Ras or Raf/Mek/Erk antagonist in a carrier which may include excipients, diluents or other pharmaceutically active compounds. Such compositions may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

The activity of the compositions of the invention in vertebrate animals may be assessed using experimental animal models of disorders in which muscle atrophy is present. For example, the activity of the compositions may be tested for their effect in the hindlimb immobilization model described herein in Example 2. Alternatively, the activity of the compositions may be assessed in animals using

experimental animals in which hypertrophy can be measured. For example, the activity of the compositions may be tested for their effect on muscles undergoing exercise-induced hypertrophy, or compensation-induced hypertrophy. Alternatively, the muscle may be assessed in control animals as compared to animals treated with the experimental compositions, to determine if the treated animals exhibit skeletal muscles hypertrophy as a result of their treatment. Data obtained from cell culture assays and animal studies can be used in formulating a range of dosages for use in vertebrate animals, including humans. The dosage of the compositions of the invention should lie within a range of serum circulating concentrations with little or no toxicity. The dosage may vary within this range depending on the dosage form employed and the route of administration.

In another embodiment, the present invention provides a method of identifying an agent that inhibits atrophy or causes hypertrophy in muscle cells, comprising creating muscle cells that express constitutively active mutants of Ras or Raf/Mek/Erk, contacting said muscle cells with an agent to be tested; and screening for cells which do not undergo atrophy or in which the amount of atrophy is reduced as compared to control cells.

In one embodiment, the present invention provides a method of identifying an agent that inhibits atrophy or causes hypertrophy in muscle cells, comprising preparing an *in vitro* assay for Ras or Raf/Mek/Erk activity, contacting the Ras or Raf/Mek/Erk with an agent to be tested; and screening for agents which block the Ras or Raf/Mek/Erk activity.

Another preferred embodiment of the present invention provides a method of identifying a gene encoding a gene-product that inhibits atrophy or causes hypertrophy in muscle cells, comprising preparing muscle cells expressing constitutively active mutants of Ras or
5 Raf/Mek/Erk; introducing into these muscle cells a gene to be tested, under conditions in which said test gene encodes a product, measuring the amount of atrophy in said test-gene encoding muscle cells and comparing the amount of atrophy in said test-gene encoding cells with the amount of atrophy in the muscle cells in which the test gene has
10 not been introduced, wherein a smaller amount of atrophy in the test-gene encoding muscle cells indicates that the test gene product inhibits the Ras or Raf/Mek/Erk pathway and therefore inhibits atrophy in cells under conditions in which such atrophy is occurring.

15 Cells useful for expressing constitutively active mutant forms of Ras or Raf/Mek/Erk include any and all muscle cells that can be maintained in culture and that can be engineered to express a heterologous nucleic acid. The cells may be primary cultures or established cell lines. Suitable muscle cells include myoblasts, for example the C2C12 cell
20 line as described in Bains, et al. (1984) Mol. Cell. Biol. 4:1449-1553, the disclosure of which is incorporated herein by reference. Other suitable muscle cells include Sol8 cells, described by Glass et al. (1997) Proc. Natl. Acad. Sci. USA 16:8848, and L6 cells, described by Ringentz et al. (1978) Exp. Cell Res. 113:233, the disclosures of which
25 are incorporated herein by reference.

Cells useful for making cultured muscle cells expressing the Ras or Raf/Mek/Erk genes also include non-muscle cells which may be differentiated into skeletal muscle cells; an example of this type of

cell is an embryonic stem-cell, which can be differentiated into a skeletal muscle cell by insertion of the muscle-specific transcription factor, MyoD, as described by Shani et al. (1992) Symp. Soc. Exp. Biol. 46:19. Another example of a non-muscle cell which can be differentiated along a skeletal muscle pathway is a rhabdomyosarcoma tumor cell, which can be differentiated by contact with retinoic acid, as described by Gabbert et al. (1988) Cancer Res. 48:5264.

The constitutively active mutant forms of the Ras or Raf/Mek/Erk nucleic acids under the control of suitable transcriptional and translational regulatory sequences can be introduced into the cell by methods known in the art including, for example, transformation, transfection, infection, transduction and injection. In a preferred embodiment, the Ras, Raf, Mek or Erk gene is contained in an expression vector under the control of a suitable promoter to effect expression in muscle cells. Preferred promoters include skeletal actin, such as human skeletal actin (HSA), chicken skeletal actin (CSA), mouse skeletal actin (MSA), rat skeletal actin (RSA); muscle creatine kinase (MCK); MyoD; MRF4; myogenin; dystrophin; utrophin; MuSK; myosin light chain (MLC1/3); myosin heavy chain (MHC). Other non-muscle specific promoters include cytomegalovirus (CMV); LTR (long-terminal repeat, found in retroviruses including the Moloney Murine Leukemia Virus - MuLV); HTLV-1 (the promoter for Human T-cell leukemia virus 1); late, middle, or early Adenovirus.

The amount of atrophy in cells may be measured by quantitation of diameter, protein amount, or by activation of the p70S6 kinase or Phas-1 protein, which stimulates protein synthesis. In transgenic organisms, muscle atrophy may be measured as described in Example 2
5 herein.

In yet another preferred embodiment, Ras, Raf, Mek or Erk protein is expressed in an *in vitro* assay, such that agents can be screened which inhibit these signaling molecules. Alternatively, *in vitro* binding
10 assays may be utilized to identify agents which block the binding of Ras, Raf, Mek or Erk to downstream signaling components, using, for example, technology as described in End, et al. (1993) J. biol. Chem. 268(14):10066-10075.

15 The expression vector containing the constitutively active mutant form of the Ras, Raf, Mek or Erk nucleic acid under the control of a suitable promoter is introduced into the cell by known methods, for example liposome-mediated transfection, calcium phosphate-mediated transfection, DEAE-dextran transfection, naked DNA transfection,
20 microinjection, electroporation, retroviral-mediated infection, adenoviral-mediated infection, or adeno-associated viral-mediated infection. The constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid can be introduced into the cell stably or transiently. Methods for introducing heterologous nucleic acids into eukaryotic
25 cells are described in numerous laboratory manuals including, for example, DNA Cloning: A Practical Approach, vols. I-III (1985) Glover, ed., IRL Press Limited, Oxford, and Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor, NY.

In a preferred embodiment, the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid is inserted into a retroviral vector, for example, as described by Pear et al. (1993) Proc. Natl. Acad. Sci. USA 90:8392, incorporated herein by reference. In this

5 embodiment, the viral LTR promoter controls the transcription of the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid. The vector is transiently transfected into a retroviral packaging line, and the resulting recombinant virus which contains the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic
10 acid is harvested, as described by Pear et al., id. The recombinant virus is then used to infect myoblasts as described by Hoffman et al. (1996) Proc. Natl. Acad. Sci. USA 93:5185, incorporated herein by reference.

C2C12 cells, as an example of skeletal muscle cells, expressing constitutively active mutant forms of Ras, Raf, Mek, or Erk, can be
15 maintained in the undifferentiated state by growing them in tissue culture media containing at least 10% fetal calf serum, or they can be differentiated into skeletal muscle myotubes by growing them in media containing 2% horse serum. The necessary tissue culture methods are known to those of ordinary skill in the art. C2C12 cells are described
20 in Bains et al. (1984) Mol. Cell Biol. 4:1449, incorporated herein by reference.

Muscle cells that express the constitutively active mutant forms of the Ras, Raf, Mek, or Erk nucleic acid may be contained within, or obtained
25 from, a transgenic organism. The term transgenic organism as used herein includes muscle cell-containing multicellular organisms that have a foreign gene, and in particular the constitutively active mutant forms of the Ras, Raf, Mek, or Erk nucleic acid, incorporated into their genome. Transgenic organisms contemplated in accordance with the

present invention include worms, birds, chickens, turkeys, flies and non-human mammals such as, for example, mice, rats, dogs, rabbits, sheep, pigs, sheep, goats, horses and cattle. Transgenic worms and mice are particularly preferred for research use in accordance with the present invention.

In the transgenic organisms of the present invention, the coding region of the constitutively active mutant forms of the Ras, Raf, Mek, or Erk nucleic acid under the control of a muscle-specific regulatory element is integrated into the genome of the host organism. The muscle-specific regulatory element directs expression of constitutively active mutant forms of the Ras, Raf, Mek, or Erk nucleic acid to provide expression of these nucleic acids in muscle cells of the host organism, and may be native to the host organism, or heterologous to the host organism. Preferred muscle-specific regulatory elements useful for the present invention are selected for optimal expression in the particular host. Preferred muscle-specific regulatory elements for use in non-human transgenic mammals include the human skeletal actin (HSA) promoter (Brennan et al. (1993) J. Biol. Chem. 268:719, incorporated herein by reference) and the muscle creatine kinase (MCK) promoter (Johnson et al. (1989) Mol. Cell. Biol. 9:3393, incorporated herein by reference).

The transgenic organisms of the present invention are made by transferring a transgene DNA construct comprising the coding region of the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid operably linked to a muscle-specific promoter into a host organism such that the transgene is integrated into the genome of the host organism. Methods for making transgenic non-human organisms

are known in the art and include, for example, DNA microinjection, embryonic stem (ES) cell transfer, retroviral infection, blastomere-embryo aggregation, teratocarcinoma cell transfer, electrofusion, nuclear transplantation, and spermatozoa-mediated transfer. The methods are reviewed, for example by Pinkert et al., "Transgenic Animal Modeling" in Molecular Biology and Biotechnology, Meyers, ed., VCH Publishers, Inc., New York, 1995, pp. 90-907 and numerous laboratory manuals including, for example, Hogan et al. (1994) Manipulating the Mouse Embryo: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Plainview, New York, the disclosures of which are incorporated herein by reference. In a preferred embodiment of the present invention, the transgenic non-human organisms are produced by microinjection. A transgenic organism contemplated in accordance with the present invention includes, but is not limited to, a transgenic fly, worm, bird, chicken, turkey, mouse, rat, dog, cat, rabbit, sheep, pig, goat or horse. In a preferred embodiment of the present invention, the transgenic organism is a transgenic mouse. Methods for making transgenic worms, in particular Caenorhabditis elegans, are also known in the art and disclosed, for example by Mello et al. (1991) EMBO.J. 10:3959.

In a preferred embodiment of the present invention, the transgenic organism is a transgenic mouse whose germ cells and somatic cells contain a transgene comprising the coding region of the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid operably linked to a muscle-specific promoter, whereby the constitutively active mutant form of Ras, Raf, Mek, or Erk protein is thus produced in the muscle cells of the transgenic organism. The transgenic mouse is preferably made by transfection of ES cells with a vector comprising

the transgene, injection of the transfected ES cells into host blastocysts into pseudopregnant foster mother hosts, followed by embryonic development and birth of the transgenic mice. The constitutively active mutant form of Ras, Raf, Mek, or Erk nucleic acid is expressed in muscle cells of the transgenic mice to provide the constitutively active mutant form of the Ras, Raf, Mek or Erk protein in the muscle cells.

In another preferred embodiment, the transgenic mouse is made by injection of a vector comprising the transgene directly into the pronucleus of a fertilized egg, as described in Hogan et al., supra. The injected egg is inserted into pseudopregnant foster mother hosts, followed by embryonic development and birth of the transgenic mouse.

The presence of constitutively active mutant forms of Ras, Raf, Mek or Erk protein can also be assessed using antibodies specific for these proteins, or by antibodies specific for an epitope-tag which is genetically inserted into the vectors encoding these proteins.

In methods for the identification of an agent that inhibits muscle cell atrophy, the agent may be contacted with the muscle cell expressing constitutively active mutant forms of Ras, Raf, Mek or Erk by methods known in the art. For cells in culture or cells obtained from transgenic organisms, the cell may be contacted with the agent by, for example, direct application. The agent may be modified or contained in a delivery vehicle to facilitate entry into the cell. The agent may be isolated and purified, or it may present in a sample or composition to be subjected to further purification subsequent to a positive result in the present method. For example, the agent may be contained in a cell

lysate, conditioned cell culture media, or a library of synthetic or naturally occurring compounds. For muscle cells present in a transgenic organism, the cells may be contacted with the agent by delivering the agent by methods known in the art, for example by ingestion, parenteral administration, or direct application to tissue surfaces, and may be present in a composition comprising a carrier or diluent. Agents that may be tested in the method of the present invention include, for example, organic and inorganic molecules such as proteins, peptides, lipids, carbohydrates, nucleic acids, including antisense, metals, salts and so on.

In methods for identifying a gene, the gene product of which inhibits atrophy in muscle cells in the presence of constitutively active mutant forms of Ras, Raf, Mek or Erk, the gene to be tested may be introduced into the muscle cell by methods known in the art and described hereinabove for inhibition of the Ras, Raf, Mek or Erk signaling effects. The gene is under the control of a promoter, as described hereinabove, and may be present in an expression vector. Pools of genes, for example cDNA libraries, may be tested for their ability to interfere with proteolysis. Upon identification of a gene pool that inhibits proteolysis, the pool may be progressively divided and tested until a single gene is identified.

In methods for identifying a gene, the expression of which inhibits atrophy in muscle cells containing constitutively active mutant forms of Ras, Raf, Mek or Erk, genetic mutations may be introduced into a gene in muscle cells by methods known in the art. Methods of directed mutagenesis are well-known in the art, and may be found in laboratory manuals including for example, Directed Mutagenesis: A Practical

Approach (1991) McPherson, ed., Oxford University Press, Oxford. In a preferred embodiment, random mutagenesis is accomplished using the chemical EMS, which can be accomplished by those skilled in the art. An example can be found in Chanal, et al., (1997) Genetics 146:20.

5 Methods to induce mutations are reviewed by Anderson (1995) Methods Cell Biol. 48:31.

The methods of the present invention are useful for the identification of agents, genes and gene products that interfere with atrophy in

10 muscle cells containing constitutively active mutant forms of Ras, Raf, Mek or Erk. The agents, genes and gene products identified by the present methods are useful for the treatment and prevention of muscle atrophy.

15 References cited hereinabove are incorporated herein in their entirety.

The following non-limiting examples serve to further illustrate the present invention.

20 EXAMPLES

Example 1

The Effect of Activation and Blocking of Raf in Muscle Cells

25 To test the effects of activating and blocking Raf in muscle cells, a strain of myoblasts called C2C12 cells were transfected with DNA expression vectors containing either a constitutively active Raf gene [Heidecker, et al. (1990) Mol. Cell. Biol. 10:2503; Samuels, et al. (1993) Mol. Cell. Biol. 13:6241; Morrison, et al. (1997) Curr. Opin. Cell Biol.

9:174] or a dominant negative Raf gene [Bruder, et al. (1992) Genes Dev. 6:545], or, as a control, the expression vector without any gene inserted. A "dominant negative" construct is able to block activity of the endogenous gene product; a "constitutively active" construct
5 encodes a mutated form of the gene which is always in the activated state. These constructs were made using standard molecular biology techniques, and were then stably transfected into C2C12 cells, using the calcium phosphate transfection method. Transfected cells were selected by antibiotic selection. The resulting myoblasts were then
10 grown to confluence, and differentiated into "myotubes", which are multi-nucleated muscle cells, similar to actual muscle fibers.

Myotubes expressing the dominant negative form of Raf were found to be much larger than control myotubes - the myotubes were longer, and
15 had broader diameters. Analysis of Phas-1, a molecule involved in protein synthesis [Xu, et al. (1998) J. Biol. Chem. 273(8):4485-4491] demonstrated that inhibition of Raf causes an increase in Phas-1 activation, thus demonstrating that blocking Raf increases muscle protein synthesis.

20 Myotubes expressing the constitutively active form of Raf were found to be significantly thinner than control myotubes. Analysis of Phas-1 in these myotubes demonstrated that activation of Raf causes a decrease in Phas-1 activation, demonstrating that activation of Raf
25 decreases muscle protein mass, and that inhibition of Raf may be used to decrease or prevent muscle atrophy.

In addition, C2C12 myotubes were treated with the Mek 1/2 inhibitor known as PD98059 [Dudley, et al. (1995) Proc. Natl. Acad. Sci., 92:

7686], and compared to differentiated cells expressing dominant negative Raf. Cells treated one day post-differentiation with 3 mM of the Mek-inhibiting drug were indistinguishable from dominant negative Raf cells.

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Example 2

Animal Model for Testing for Muscle Atrophy

To test for muscle atrophy, the ankle joint of rodents (mice or rats) are immobilized at 90 degrees of flexion. This procedure induces atrophy of the muscles with action at the ankle joint (e.g. soleus, medial and lateral gastrocnemius, tibialis anterior) to varying degrees. A reproducible amount of atrophy can be measured in hindlimb muscles over a 14-day period.

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The immobilization procedure may involve either casting (mice) or pinning the ankle joint (rats). Rodents are anesthetized with ketamine/xylazine and the right ankle joint is immobilized. In rats, a 0.5 cm incision is made along the axis of the foot, over the heel region. A threaded screw (1.2 x 8mm) is then inserted through the calcaneus and talis, into the shaft of the tibia. The wound is closed with skin glue. In mice, the ankle joint is fixed at 90 degrees with a light weight casting material (VET-LITE) around the joint. The material is soaked in water and then wrapped around the limb. When the material dries it is hard, but light in weight.

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At seven and 14 days following the immobilization, animals are anesthetized and killed by cervical dislocation. The tibialis anterior (TA), medial gastrocnemius (MG), and soleus (Sol) muscles are removed

from the right (immobilized) and left (intact) hindlimbs, weighed, and frozen at a fixed length in liquid nitrogen cooled isopentane. A cohort of control animals which are the same weight and age as the experimental animals are also killed and the muscles removed, weighed and frozen. The amount of atrophy is assessed by comparing the weight of the muscles from the immobilized limb with the weight of the muscles from the control animals.

Example 3

10 Preparation of Muscle-Specific Eukaryotic Expression Constructs for Constitutively Active Ras and Constitutively Active Raf

DNA encoding constitutively active Ras and DNA encoding constitutively active Raf, was cloned into eukaryotic expression vectors by standard molecular biology techniques. Briefly the DNA encoding a constitutively active RasV12, and DNA encoding the COOH terminal of Raf (RafCT), which is constitutively active when expressed, was gel-purified and cloned into eukaryotic expression vectors containing the Muscle Creatine Kinase (MCK) promoter. These vectors were called MCK RasV12 or MCK RafCT, respectively. MCK promoters have been previously shown to drive muscle-specific expression of transgenes in mice [Johnson et al. (1989) Mol. Cell. Biol. 9:3393].

Example 4

25 Transfecting C2C12 Cells

Murine C2C12 myoblasts were used as a means of studying the transgenes in cell culture. To achieve Ras and Raf gene expression, vectors using the murine muscle creatine kinase (MCK) gene promoter

as described in Example 3 were transfected into C2C12 cells by calcium phosphate precipitation. To allow for the positive selection of transgenic C2C12 cells, using the drug G418 (Gibco), the MCK- RasV12, and RafCt vectors were co-transfected with a plasmid containing a LTR gene promoter driving a *neo* gene. Expression of this plasmid allows a cell to resist the killing effect of the drug G418; thus, only cells which have been successfully transfected will survive when treated with G418.

The transfected C2C12 cell colonies were harvested, trypsinized, and re-plated for growth in individual wells of a 24-well culture plate. Control C2C12 cells, C2C12 cells containing the MCK RasV12 construct and C2C12 cells containing the MCK RafCT construct were grown to confluence. The serum level was dropped to allow fusion of the myoblasts into differentiated muscle cells. C2C12 cells differentiate into multi-nucleated myotubes. Phenotypes of resultant myotubes were quantitated by diameter, protein amount, and by activation of the Phas-1 protein, which stimulates protein synthesis.